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Hookworm Excretory/Secretory Products Induce Interleukin-4 (IL-4)$^+$ IL-10$^+$ CD4$^+$ T Cell Responses and Suppress Pathology in a Mouse Model of Colitis

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Evidence from human studies and mouse models shows that infection with parasitic helminths has a suppressive effect on the pathogenesis of some inflammatory diseases. Recently, we and others have shown that some of the suppressive effects of hookworms reside in their excretory/secretory (ES) products. Here, we demonstrate that ES products of the hookworm *Ancylostoma caninum* (AcES) suppress intestinal pathology in a model of chemically induced colitis. This suppression was associated with potent induction of a type 2 cytokine response characterized by coexpression of interleukin-4 (IL-4) and IL-10 by CD4$^+$ T cells, downregulation of proinflammatory cytokine expression in the draining lymph nodes and the colon, and recruitment of alternatively activated (M2) macrophages and eosinophils to the site of ES administration. Protease digestion and heat denaturation of AcES resulted in impaired induction of CD4$^+$ IL-4$^+$ IL-10$^+$ cell responses and diminished ability to suppress colitis, indicating that protein component(s) are responsible for some of the immunosuppressive effects of AcES. Identification of the specific parasite-derived molecules responsible for reducing pathology during chemically induced colitis could lead to the development of novel therapeutics for the treatment of human inflammatory bowel disease.

The existence of parasitic helminths predates that of humans (1), and it is believed that some parasite-host interactions, such as those of gastrointestinal helminths and their vertebrate hosts, have developed in such a way that benefits both the parasite and the host. This phenomenon is embodied by the "hygiene hypothesis," which suggests that the elimination of pathogens such as helminths from people living in the developed world has predisposed the immune system to respond inappropriately to self and otherwise innocuous environmental antigens, culminating in increased incidences of allergic and autoimmune diseases (2).

Inflammatory bowel disease (IBD) is a term used to describe two chronic inflammatory diseases, ulcerative colitis (UC) and Crohn's disease (CD). IBD is characterized by a dysregulation of the mucosal immune response to intestinal bacteria, resulting in chronic inflammation of the gastrointestinal tract, pain, diarrhea, and vomiting (3). At present, treatment methods range from lifelong use of immunomodulatory drugs (e.g., corticosteroids) to surgery; however, therapies involving deliberate human infection with helminths have been proposed as an alternative treatment method for these chronic diseases. Multiple clinical trials in humans have demonstrated that exposure to gastrointestinal parasites can significantly reduce the severity of intestinal inflammation in humans with UC (4) and CD (5). These clinical trials utilized the pig whipworm *Trichuris suis*, where infection is short-lived in humans and requires repeated administration of larvae to maintain the infection (6). Recent clinical trials performed by our laboratory have focused on the potential use for helminths that persist within the intestine, such as the hookworm *Necator americanus*, to alleviate intestinal inflammation associated with celiac disease (5, 11, 21). This hookworm-based therapy resulted in the suppression of proinflammatory anti-gliadin immune responses (21) and the induction of systemic and mucosal type 2 cytokine responses (7), although overt suppression of clinical disease was not observed (8). Although the potential benefits of parasite-derived therapies for IBD and other autoimmune diseases are apparent, the safety of such approaches has been questioned (9, 10), and a "fear factor" reaction by the public, as well as logistical concerns for scale-up, may preclude their widespread use.

The mechanism of parasite-mediated suppression of inflammatory immune responses has been investigated in a number of mouse models of disease, with roles described for cross-regulation of inflammatory Th1 responses by parasite-derived Th2 responses (11), regulatory T cells (12–14), and suppressive macrophages (15, 16). Helminths secrete proteins that modulate and/or skew immune responses (17, 18), suggesting that "helminth therapy" for autoimmunity could take the form of soluble molecules derived from helminths rather than an active infection (19).

Here we show that the administration of *Ancylostoma caninum* excretory/secretory products (AcES) limits intestinal pathology and proinflammatory cytokine expression during dextran sodium sulfate (DSS)-induced colitis. Injection of AcES in mice induces a

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robust antigen-specific type 2 cytokine response, including the emergence of a distinct $CD4^+$ T cell population that coexpresses IL-4 and IL-10 and the recruitment of macrophages and eosinophils to the site of injection. Denaturation of AcES resulted in a loss in the protective effect during colitis, suggesting that the immunomodulatory properties of AcES are at least partly due to a protein constituent.

MATERIALS AND METHODS

Production of *A. caninum* ES. *A. caninum* adult worms were cultured in serum free medium containing 100 U of penicillin/μl and 100 μg of streptomycin/ml (pen/strep) for 24 h. The supernatant (AcES) was collected, filter sterilized through a 0.22-μm-pore-size filter (Pall), and concentrated and buffer exchanged to phosphate-buffered saline (PBS) using a 10-kDa spin column (Pall). Removal of lipopolysaccharide from AcES and ovalbumin (OVA; Sigma) was then performed using one of two methods, Endotrap Blue (Hyglos) according to the manufacturer's instructions or Triton X-114 (Sigma) as previously described (20) with some minor changes. Briefly, AcES was incubated with 5% Triton X-114 at 4°C on a rotating wheel for 30 min, followed by heating to 37°C for 10 min and centrifugation at 1,600 × *g* for 15 min at room temperature. The upper endotoxin-depleted phase was collected, and the process was repeated twice to ensure thorough removal of endotoxin. A *Limulus* amebocyte lysate (Lonza) assay was used to ascertain the adequate removal of endotoxin, and the protein concentration was calculated by using a micro-BCA protein assay kit (Pierce). Some experiments used boiled and trypsinized AcES (bES) as a control. Briefly, AcES was digested with 1 μg of trypsin (Sigma)/ml at 37°C for 24 h, followed by boiling at 95°C for 15 min to denature both trypsin and the AcES protein constituents.

Mice. Female 6- to 10-week-old C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia) and were housed according to Australian animal rights and regulations standards. Mice received food and water *ad libitum*. All injections were administered via the intraperitoneal (i.p.) route without adjuvant. In some experiments, mice received i.p. injections of PBS or 1 to 25 μg of AcES, bES, or OVA at various time points as indicated in the text. All procedures involving mice were approved by the James Cook University Animal Ethics Committee.

DSS-induced colitis. A 3.5% (wt/vol) solution of dextran sodium sulfate (DSS; 36,000 to 50,000 molecular weight; MP Biomedicals) was administered to mice as a substitute for normal drinking water. The mice were weighed and scored daily to assess disease progression based on a modified scoring system (21). Mice were scored on weight (percent change; 0 to 4), the level of fecal consistency (0 to 4), rectal bleeding (0 to 2), and general appearance (0 to 3) for a daily score out of a total of 13.

Histopathology. Upon termination of the experiment the mouse colons were given a macroscopic score for severity of adhesion (0 to 3), ulceration (0 to 3), wall thickening (0 to 3), and edema (0 to 3) for a total possible score of 12 as previously described (22). A small piece of the proximal colon was fixed in 4% formaldehyde for histological processing. Cross-sections of the colons were stained with hematoxylin and eosin (H&E) for microscopic visualization of inflammation. Histological scoring of the cross-sections was performed in a blinded fashion using a modified scoring system (23). Colon cross-sections were assessed for number of ulcers (no ulcers = 0, 1 ulcer = 1, 2 ulcers = 2, 3 ulcers = 3, and >3 ulcers = 4). Each ulcer was ~200 μm in length; where ulceration was bigger than this, scoring was performed in 200-μm intervals. Epithelium integrity was scored follows: 0 = normal morphology, 1 = loss of goblet cells in 1 area, 2 = loss of goblet cells in more than one area, 3 = loss of crypts in 1 area, and 4 = loss of crypts in more than one area. Cellular infiltrate was scored as follows: 0 = no infiltrate, 1 = infiltrate around crypt bases, 2 = infiltrate reaching to muscularis mucosae, 3 = extensive infiltration reaching the muscularis, and 4 = infiltration of the submucosa with edema. Finally, lymphoid follicles were scored as none = 0, 1 = 1, 2 = 2, 3 = 3, >3 = 4. Together, these criteria could achieve a total possible score of 16.

Cell preparation and cytokine analysis. Peritoneal cells were collected by washing the peritoneal cavity with 10 ml of ice-cold complete medium (RPMI 1640 plus 10% heat-inactivated fetal calf serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine; Invitrogen). Splenocyte restimulations and cytokine assays were performed as previously described (24). Briefly, spleens were macerated through 70-μm-pore-size nylon filters (BD Biosciences), and red blood cells were lysed using red blood cell lysis buffer (Sigma). Splenocytes were cultured in triplicate in flat-bottom 96-well plates (10^6 cells/well) either in medium alone or in medium supplemented with AcES (10 μg/ml), OVA (10 μg/ml), or anti-CD3 (1 μg/ml) for 72 h at 37°C and 5% CO_2. Colon lysates were produced by flushing colons with PBS and placing a small piece of known weight into 1 ml of PBS and lysing on a TissueLyser (Qiagen) with the use of a metal bead. Cell-free supernatants were removed and concentrations of IL-4, IL-5, IL-10, gamma interferon (IFN-γ), IL-17A, and tumor necrosis factor alpha (TNF-α) were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA; OptEIA kits; BD Biosciences).

Flow cytometry. Peritoneal cells were stained for CD11c-FITC (clone HL3), SIGLEC-F-PE (clone E50-2440) (BD Biosciences), and F4/80-APC (clone BM8; Caltag/Invitrogen), acquired on a FACSCanto flow cytometer (BD Biosciences), and analyzed using FlowJo software (TreeStar). Intracellular cytokine stains were performed on spleen and lymph node cells. Prior to staining, the cells were cultured for 4 h at 37°C and 5% CO_2 in complete medium containing phorbol myristate acetate (500 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml). The cells were stained for CD4-FITC (clone RM4-5; BD Biosciences) and then permeabilized with Fix/Perm buffer (BD Biosciences) and stained for IL-4-PE (clone 11B11; BD Biosciences), IL-10-APC (clone JES5-16E3; eBioscience), and IFN-γ-eF450 (clone XMG1.2; eBioscience).

RNA extraction and real-time PCR. For peritoneal cells, 10^6 cells were pelleted by centrifugation and resuspended in 1 ml of TRIzol (Invitrogen). Similarly, a small 0.5-cm piece of colon was washed in PBS, placed in 1 ml of TRIzol, and macerated on a TissueLyser (Qiagen) for 10 min with the use of a metal bead. Total RNA extraction was performed by phenol-chloroform separation according to the manufacturer's instructions. After treatment of RNA with RQ1 DNase (Promega), first-strand cDNA was produced with random hexamers (Invitrogen) from 0.5 to 1 μg of total RNA by using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The levels of transcription were measured by comparing cross-threshold values to a standard curve made of a pool of all samples. Samples were tested in dilutions of up to 1:600 using SYBR green (Applied Biosystems/Qiagen). A Rotor Gene 6000 (Qiagen) was used for real-time thermal cycling. Melting-curve analysis was used to confirm that a single product had been amplified. All genes were normalized for levels of transcription relative to the housekeeping gene β-actin. All primers were purchased from Sigma-Aldrich and were diluted to a 10 μM final concentration. The primers used were as follows: β-actin, sense (TGGAATCCTGTGGCATCCATGAAAC) and antisense (TAAAACGCAGCTCAGTAACAGTCCG); Fizz-1, sense (GTCCTGGAACCTTTCCTGAG) and antisense (AGCTGGATTGGCAAGAAGTT); Ym1, sense (CTGAGAAGCTCATTGTGGGA) and antisense (CTCAGTGGCTCCTTCATTCA); Arg-1, sense (CAGAAGAATGGAAGAGTCAG) and antisense (CAGATATGCAGGGAGTCACC); NOS-2, sense (ACCTTGTTCAGCTACGCCTT) and antisense (CATTCCCAAATGTGCTTGTC); IL-6, sense (CCGGAGAGGAGACTTCACAG) and antisense (TCCACGATTTCCCAGAGAAC); IL-17A, sense (CCTCCAGAATGTGAAGGTCA) and antisense (CTATCAGGGTCTTCATTGCG); and IFN-γ, sense (AGCTCTTCCTCATGGCTGTT) and antisense (TTTGCCAGTTCCTCCAGATA).

Statistical analyses. All data were analyzed with GraphPad (version 5; Prism). When three or more groups were compared, one-way analysis of variance (ANOVA) was used with a Bonferroni post-test with a 95% confidence interval to compare all columns. When the effect of a treatment over time was compared for different treatment groups, two-way ANOVA was used with a Bonferroni post-test to compare replicate means over

time. P values of <0.05 were considered significant. When only two groups were compared, a Mann-Whitney test was used. All results stated in the text are means ± the standard errors of the mean (SEM). None of the figures presented here are pooled from multiple runs, and all data are representative of at least three repeat experiments.

RESULTS

AcES products protect mice from DSS-induced colitis. Ingestion of DSS via the drinking water by mice injected i.p. with vehicle (PBS) or a control protein (OVA) caused rapid weight loss beginning at day 5 posttreatment compared to mice receiving normal drinking water (Fig. 1A). In contrast, i.p. administration of AcES protected against DSS-mediated weight loss (Fig. 1A). Upon euthanasia, the colons were scored for pathology on a macroscopic level (Fig. 1B), and H&E-stained tissue sections scored at a microscopic level (Fig. 1C), showing that AcES-treated mice had significantly lower colon pathology than control mice. Representative transverse sections of the colons demonstrate that mice receiving DSS and either PBS or OVA control treatments had increased cellular infiltrate and edema in the submucosa, whereas mice that had been treated with AcES had markedly less infiltrate and swelling (Fig. 1D).

AcES reduces levels of proinflammatory cytokines associated with pathology in the draining lymph nodes and the colon. We next addressed the impact of AcES administration on the expression levels of proinflammatory cytokines associated with DSS-induced pathology. Mice received 3.5% DSS in their drinking water for 8 days and received either a vehicle injection of PBS or 1, 10, or 25 μg of AcES daily i.p. Mesenteric lymph node (MLN) and spleen cells were polyclonally stimulated with anti-CD3 *in vitro*, and cytokine production was measured by ELISA. The results showed a dose-dependent suppression of IFN-γ and IL-17A expression by MLN cells (Fig. 2A) and splenocytes (data not shown).

We next compared the levels of gene expression at the site of inflammation, the colon, in mice treated with either vehicle or 25 μg of AcES. Critically, expression of the proinflammatory mediators iNOS, IL-6, and IL-17A was significantly reduced by AcES treatment (Fig. 2B), and the levels of IFN-γ tended to be lower but did not reach statistical significance ($P = 0.0585$). In contrast, IL-4 and IL-10 protein levels in the colon were increased in AcES-treated mice compared to control-treated mice (Fig. 2C). Together, these data indicate that AcES causes downregulation of proinflammatory type 1/type 17 responses potentially by inducing a bias toward a type 2 or regulatory cytokine.

AcES products induce a biased Th2 cytokine response in the absence of any adjuvant or live infection. To further investigate the induction of a type 2 cytokine response in mice receiving AcES, mice were injected with either PBS alone or 10 μg of AcES every 2 days for a total of 2 weeks in the absence of any other stimulus. A 10-μg dose was chosen as pilot studies had indicated that 10 μg produced a similar Th2 response as a 25-μg dose in the absence of DSS. Analysis of intracellular cytokine staining in peritoneal lavage cells demonstrated that injection of AcES into mice caused significantly reduced frequencies of $CD4^+$ T cells that expressed IFN-γ (Fig. 3A) and significantly increased frequencies of $CD4^+$ T cells expressing IL-4 and IL-10, including a prominent population expressing both of these cytokines (Fig. 3B). AcES injection also induced populations of IL-4/IL-10 double-positive CD4 T cells in the spleen and MLN (data not shown). Significant increases in AcES-specific IL-4, IL-5, and IL-10 production by

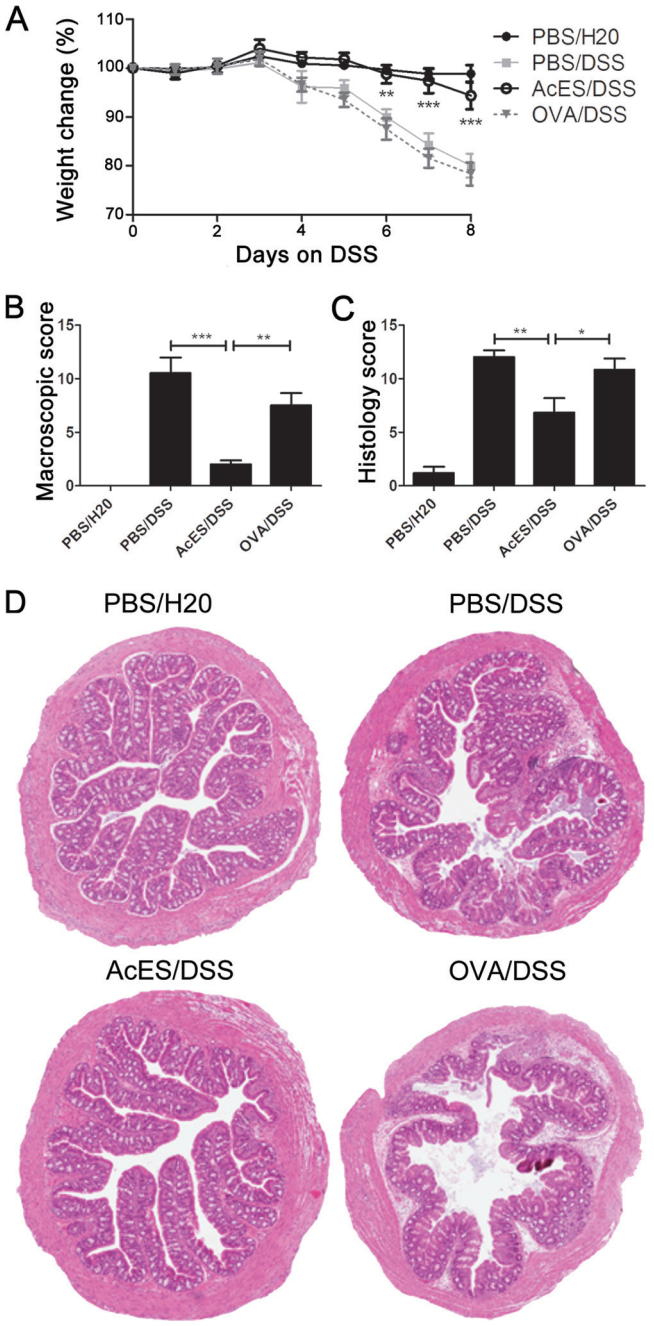

FIG 1 AcES protects mice from DSS induced colitis. Mice received 3.5% DSS in drinking water to induce colitis; test groups received 25 μg of AcES or OVA i.p. every day. (A) Weight loss graph, showing the percentages of mean weight lost compared to day 0. (B) Colons from mice were removed and assessed for levels of macroscopic inflammation based on adhesion, ulceration, edema, and wall thickening for a total possible score of 12. (C) Histology sections were assessed for levels of inflammation based on ulceration, epithelial integrity, lymphoid follicles, and cellular infiltrate for a total possible score of 16. (D) Representative transverse sections (×20 magnification) of mouse colons stained with H&E. All graphs show mean ± the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 6$). The data are representative of at least three repeat experiments.

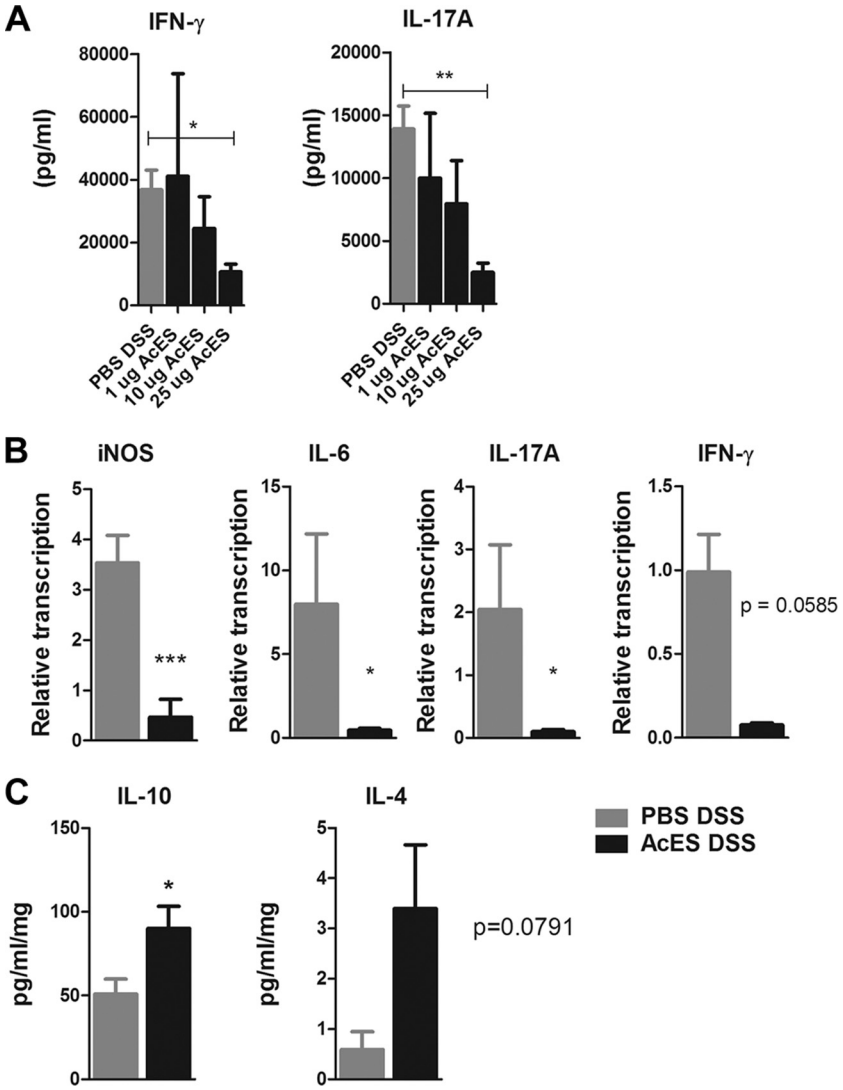

FIG 2 AcES reduces levels of proinflammatory cytokines both in the draining lymph nodes and the colon. (A) IFN-γ and IL-17A levels in culture supernatants of polyclonally stimulated MLN cells. (B) Transcription of inducible nitrous oxide synthetase (iNOS), IL-6, IL-17A, and IFN-γ by RT-PCR in the colonic tissue of mice. (C) IL-10 and IL-4 levels in colon lysates. Gray bars indicate control mice, and black bars indicate mice that received AcES. Mice received 3.5% DSS in drinking water to induce colitis; test groups received AcES i.p. daily. Where not indicated on the graph, the amount of AcES was 25 μg. All graphs show means ± the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 3$ to 6). The data are representative of at least three repeat experiments.

restimulated splenocytes were observed in AcES-treated mice compared to PBS- or OVA-treated control mice (Fig. 3C). Splenocytes from OVA-injected animals restimulated with OVA did not produce significantly elevated levels of IL-4, IL-5, or IL-10 (IL-4, 25.34 ± 4.49 pg/ml; IL-5, 0.0; IL-10, 302.1 ± 151.8 pg/ml). Finally, no significant differences were noted in the production of either TNF or IFN-γ.

AcES recruits eosinophils and M2 macrophages to the site of injection. Given the ability of AcES to induce a type 2 cytokine bias, we explored whether AcES elicits a downstream innate effector eosinophil and M2 (alternatively activated) macrophage response at the site of injection. Mice injected with AcES had significantly more cells at site of injection than control-treated animals [$(20.33 \pm 3.69) \times 10^6$ versus $(4.40 \pm 0.74) \times 10^6$, $P = 0.0055$]. Flow cytometric analysis revealed that AcES injection resulted in significantly higher frequencies and total numbers of F4/80$^+$ macrophages ($P < 0.001$) and Siglec-F$^+$ eosinophils ($P < 0.001$) in the peritoneal cavity compared to mice injected with OVA or PBS (Fig. 3D-3F).

M2 macrophages are associated with suppression of T cell responses, and anti-parasite responses (25). Therefore, we analyzed the expression of various M2 macrophage markers in peritoneal cells by reverse transcription-PCR (RT-PCR). Consistent with the macrophages recruited to the peritoneal cavity after AcES injection being of an M2 macrophage phenotype, we detected a significant increase ($P < 0.001$) in *YM1*, *FIZZ-1*, and *Arg-1* expression (Fig. 3G) in cells from mice that were injected with AcES compared to control groups. Together, these data indicate that the injection of AcES alone is able to potently modulate the immune status of mice toward a type 2 cytokine response, thereby limiting proinflammatory Th1 and Th17 cytokine responses.

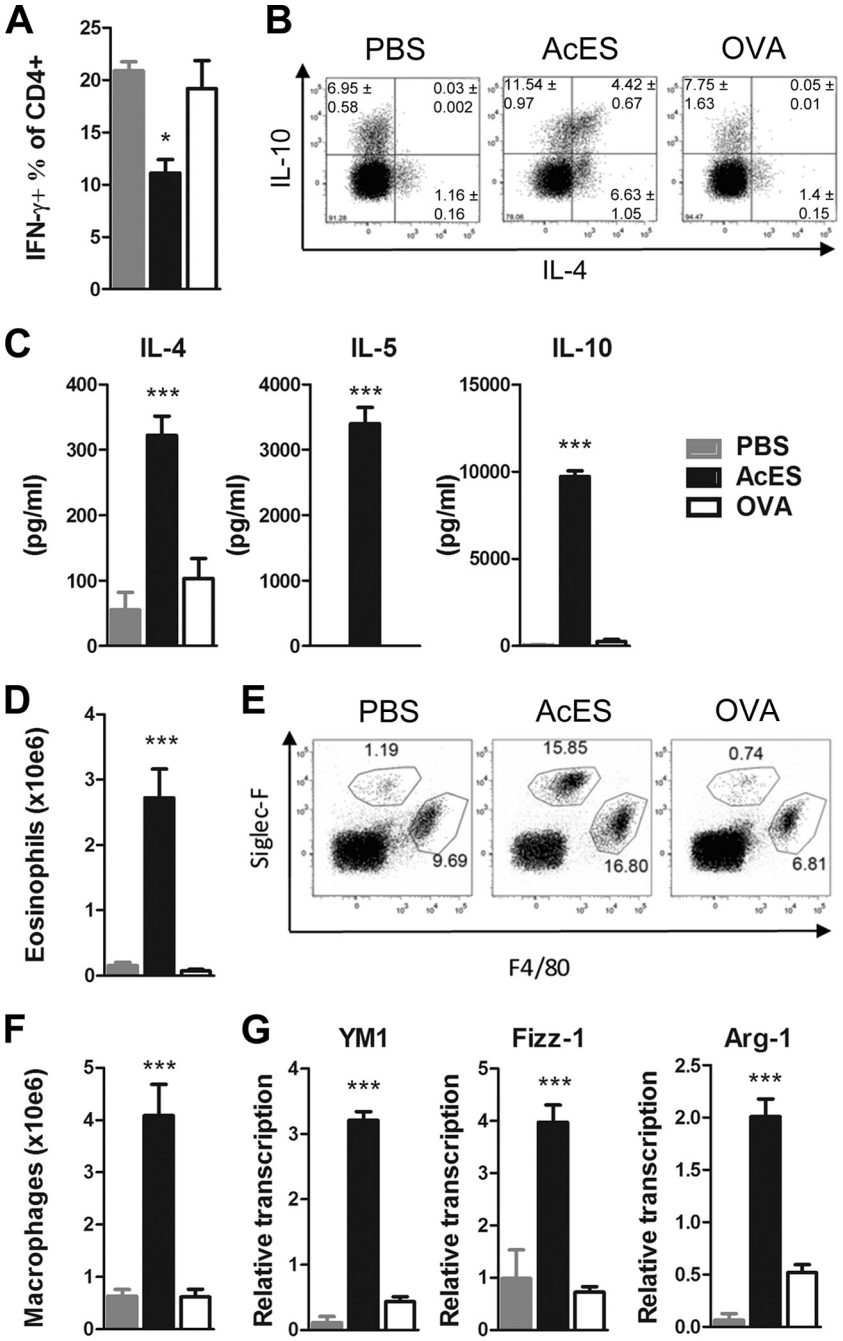

FIG 3 AcES induces an antigen-specific type 2 response, as well as a distinct population of IL-4/IL-10 double-positive CD4 T cells. (A) Frequencies of IFN-γ-producing $CD4^+$ cells in the peritoneum. (B) Representative flow cytometry plots of intracellular cytokine staining of IL-4 (*x* axis) and IL-10 (*y* axis) production by peritoneal exudate cells, gated on $CD4^+$ T cells. (C) ELISAs were performed for IL-4, IL-5, and IL-10 from supernatants of splenocytes restimulated *ex vivo* with 10 μg of AcES/ml for 72 h. (D) Number of eosinophils in the peritoneum. (E) Representative flow cytometry plots of peritoneal exudate cells, showing F4/80 (*x* axis) versus Siglec-F (*y* axis). (F) Number of macrophages in the peritoneum. (G) Transcription of M2 markers in peritoneal macrophages. Mice were injected with PBS, AcES (10 μg), or OVA (10 μg) every second day for a total of 2 weeks. Gray bars represent the PBS vehicle control group, black bars represent the AcES group, and white bars represent the OVA control group. All graphs show means ± the SEM. *, $P < 0.05$; ***, $P < 0.001$ ($n = 5$). The data are representative of at least three repeat experiments.

Denaturation and tryptic digestion of AcES diminished the induction of Th2 responses. To determine whether the factor(s) within AcES responsible for inducing type 2 cytokine responses are of a protein nature, we digested AcES with trypsin, followed by heat denaturation, a preparation we termed "boiled ES" (bES). Although the injection of AcES in DSS-treated mice resulted in characteristic reductions in IFN-γ expression and increased IL-4 and IL-10 coexpression by $CD4^+$ T cells, bES administration had a significantly diminished effect (Fig. 4A). Consistent with an impaired ability to induce a Th2 cytokine response, bES administra-

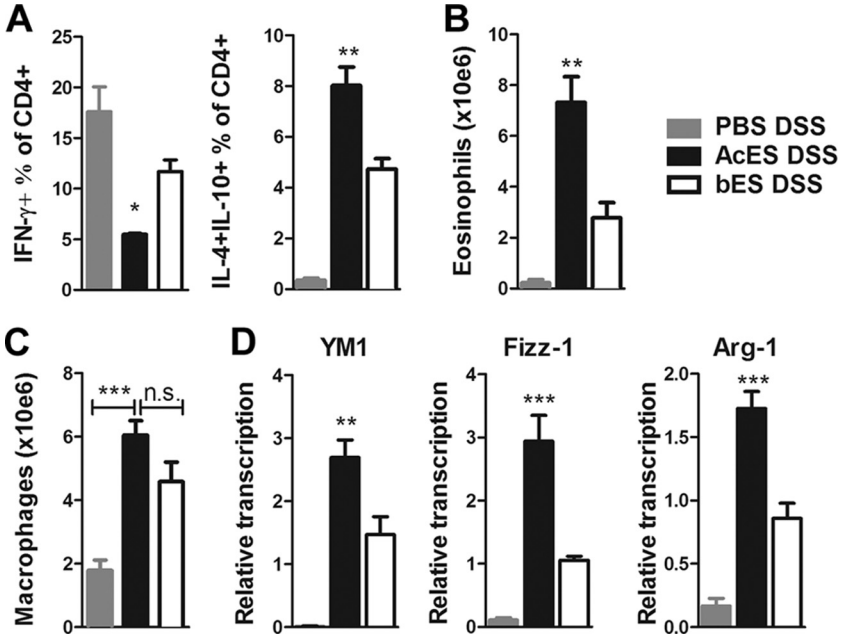

FIG 4 Denaturation and trypsin digestion of AcES leads to reduced Th2 profile. (A) Frequencies of IFN-γ-producing $CD4^+$ cells and IL-4^+ IL-10^+ CD4 T cells in the peritoneum. (B) Number of eosinophils present in the peritoneum. (C) Number of macrophages in the peritoneum. (D) Transcription of M2 markers in the peritoneum measure by RT-PCR. Mice received normal water or a 3.5% DSS solution for a period of 9 days and daily injections of either PBS, 25 μg of AcES, or 25 μg of boiled and trypsinized AcES (bES). Gray bars represent the PBS vehicle control group, black bars represent the AcES group, and white bars represent the bES group. All graphs show the mean SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 4$ to 6). The data are representative of at least three repeat experiments.

tion resulted in a less-pronounced eosinophil response (Fig. 4B) but still induced significant expansion of macrophages in the peritoneum (Fig. 4C). However, the transcription of M2 macrophage activation markers was significantly reduced in the bES mice (Fig. 4D), suggesting a reduction in alternative activation of macrophages in these mice. Similar results to these were seen when bES was administered to mice in the absence of DSS (data not shown). Hence, the ability of AcES to induce optimal type 2 cytokine responses in mice is predominantly due to heat-labile protein factors.

Protein denaturation of AcES abrogates its protective effect during DSS colitis. To assess whether the impaired ability of bES to induce a type 2 cytokine bias results in a reduced capacity to limit disease severity during colitis, we assessed weight loss and intestinal pathology in DSS-treated mice coadministered either AcES or bES. Although AcES treatment resulted in less pronounced weight loss than when mice were treated with PBS, mice injected with bES lost significantly more weight by day 7 than did mice treated with AcES (Fig. 5A). Histological analysis showed that bES-treated mice had significant edema and cellular infiltrate in the submucosa of the colon, whereas the colons of AcES-treated mice appeared relatively healthy (Fig. 5B). These data demonstrate that the component(s) of AcES that mediate protection against DSS-induced colitis is likely a protein.

DISCUSSION

Hookworms have been known survive for more than 10 years in their human hosts, and their longevity is attributed at least in part to the exquisitely refined immune-evasive strategies that they have evolved to ensure their long-term survival and propagation. While human hookworm infections exhibit some of the hallmark features of protective T helper type 2 (Th2) immune responses, including IgE and local and systemic eosinophilia, these immune responses clearly fail to protect most people from reinfection (26–28). The reason for the observed lack of an effective anti-hookworm response remains unknown, although the production of immunomodulatory ES proteins that skew or dampen immune responses to promote the long-term survival of the parasite is a likely contributing factor (29–32). In the present study, we inves-

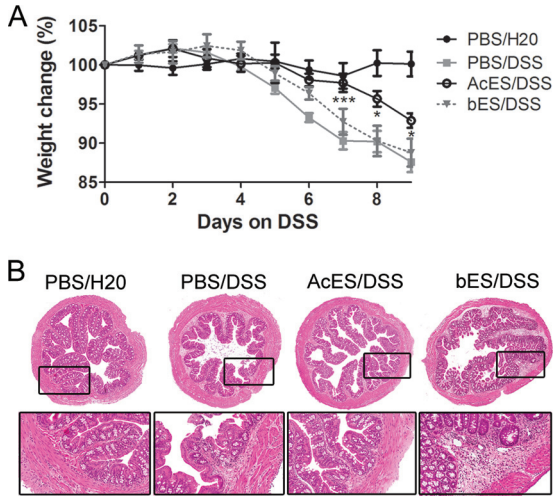

FIG 5 bES fails to protect mice against DSS-induced colitis. Mice received normal water or a 3.5% DSS solution for a period of 9 days. Mice received daily injections of either PBS, 25 μg of AcES, or 25 μg of boiled and trypsinized AcES (bES). (A) Weight loss graph, showing percentage of mean weight lost compared to day 0. (B) Low (×20)- and high (×400)-magnification histological images of H&E-stained colonic tissue. The data are representative of a minimum of three repeat experiments. Graphs show means ± the SEM. *, $P < 0.05$; ***, $P < 0.001$ ($n = 4$ to 6).

tigated the potentially beneficial properties of hookworm ES products on inflammation and the suppression of pathology associated with IBD.

Infections with live helminths from phylogenetically distant groups can protect mice against a range of autoimmune or allergic diseases (33). A growing body of literature suggests that much of this protection against inflammation is mediated by soluble molecules released by the parasites. Some examples include protection in the dinitrobenzene sulfonic acid (DNBS) model of colitis utilizing soluble somatic extracts from *Trichinella spiralis* (34) and *Hymenolepis diminuta* (35), as well as the use of ES products from *Ancylostoma ceylanicum*, a relative of *A. caninum*, in the DSS model of colitis (36). Furthermore, AcES and *Schistosoma mansoni* somatic proteins alleviated pathology associated with TNBS-mediated colitis (22). Administration of *S. mansoni* somatic proteins tended to be associated with reduced colonic transcription of inflammatory cytokines (IFN-γ, IL-12, and IL-17), increased Th2 cytokines (IL-4 and IL-5), and increased regulatory cytokines (IL-10 and transforming growth factor β) (22).

In the present study, we show that the administration of AcES in a mouse model of DSS-induced colitis prevented weight loss and significantly reduced colon pathology. The treatment of AcES alone induced a robust type 2 immune response in the draining lymph nodes and colon of mice, characterized by coexpression of IL-4 and IL-10 by $CD4^+$ T cells. Although it has been shown that the administration of AcES in a mouse model of TNBS-induced colitis protected against inflammation, no further characterization of the immunological responses were conducted (22). Here we show that both Th1 and Th17 responses characterizing colitis-induced inflammation were significantly decreased upon treatment with AcES. TNBS-mediated colitis is largely T cell dependent, whereas DSS colitis is primarily mediated by the innate cell response (21). The suppression of pathology by AcES in both models of colitis suggests a role in regulating both innate and adaptive immune responses. Indeed, we show that AcES affects elements of the adaptive (expansion of $IL\text{-}4^+IL\text{-}10^+$ $CD4^+$ T cells) and innate (expansion of M2 macrophages and eosinophils) immune responses in both diseased and healthy mice. Although we did not provide definitive proof that the IL-4/IL-10 double-positive $CD4^+$ T cells were responsible for the suppression of colitis, previous studies have shown that neutralization of both IL-4 and IL-10, but not IL-10 alone, restores IL-17 production in mice infected with *Heligmosomoides polygyrus*, suggesting a possible synergistic role of these cytokines in promoting optimal immunosuppressive activity (7).

There are numerous parallels that can be drawn between our observed effects of AcES on cytokine production in mice and in previous studies involving experimental human hookworm infections. In a recent placebo-controlled clinical trial assessing the therapeutic effect of experimental *N. americanus* infection on the immunopathogenesis of celiac disease, hookworms reduced the systemic and mucosal expression of IL-17A and IFN-γ, which are signature cytokines involved in the pathogenesis of IBD, and caused elevated Th2 cytokine responses and eosinophilia (7, 37). We observed a similar immune phenotype in mice injected with AcES, with reduced production of IL-17A and IFN-γ and elevated levels of type 2 cytokines and eosinophilia.

The ability of AcES to provoke enhanced M2 macrophage responses could also play a role in the protection against colitis due to their potential suppressive and wound healing effects (38, 39). *In vitro*-derived M2 macrophages can reduce inflammation in dinitrobenzene sulfonic acid (DNBS)-induced colitis (15), and their numbers correlate with remission of Crohn's disease in humans (15). Tapeworm extracts can also suppress classical activation of macrophages *in vitro* and suppress DNBS-mediated colitis (35). Moreover, protection against DSS-induced colitis in schistosome-infected mice was shown to be macrophage dependent; however, it did not appear to be due to M2 macrophages (16). Thus, although the suppression of M1 (inflammatory) macrophage activation and M2 macrophage activation may act through separate pathways, macrophages are clearly pivotal for exacerbation and suppression of colitis. However, denaturation of AcES (bES) did not affect recruitment of macrophages but did ablate the protection against colitis, suggesting that these cell types are not required for the anti-inflammatory properties of AcES in DSS-induced colitis. We believe that the transcription of M2 markers is dependent on IL-4 expression, and thus the reduced numbers of IL-4 expressing cells in the bES mice leads to reduced expression of M2 markers.

Finally, the protection induced by AcES in our model of colitis seemed to be entirely due to protein moieties that are sensitive to denaturation. AcES is comprised of more than 100 different proteins (40), as well as uncharacterized lipids and carbohydrates. Our study suggest that the protective properties of AcES is likely due to one or several protein components and that some of these are also necessary for the induction of the Th2 response. Ultimately, our goal is to find therapeutic specificities for each of the identified proteins of interest. However, in order to develop such molecules as therapeutics for human inflammatory diseases, the active product(s) first needs to be identified through thorough testing in animal models of disease. Some excretory molecules from other helminths, including a protein from *Trichinella spiralis* (41), phosphorylcholine from *Acanthocheilonema viteae* (42), and a glycan from *S. mansoni* (43) have already been identified as potential candidates to treat inflammatory diseases. We anticipate that AcES will be a reservoir of novel therapeutic targets for treatment of mammalian diseases.

In summary, we present data showing that a protein constituent of AcES suppresses pathology in a mouse model of IBD, correlating with a reduced inflammatory response in the intestine and a robust type 2 cytokine response, characterized by a distinct population of IL-4/IL-10 double-positive $CD4^+$ T cells. Future work will focus on defining the protective mechanisms and the specific protein components of AcES that are responsible for these effects. The recent characterization of the AcES proteome using tandem mass spectrometry will help in the identification of potential immunosuppressive factors (40). Helminths are masterful immunomodulators, and we show here that much of their suppressive capacity lies within their secreted proteins, so it is reasonable to assume that these organisms are a prime source of novel anti-inflammatory therapeutics for human diseases.

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REFERENCES

1. **Poinar G, Jr, Boucot AJ.** 2006. Evidence of intestinal parasites of dinosaurs. Parasitology **133**:245–249.
2. **Strachan DP.** 1989. Hay fever, hygiene, and household size. BMJ **299**: 1259–1260.
3. **Podolsky DK.** 2002. Inflammatory bowel disease. N. Engl. J. Med. **347**: 417–429.
4. **Summers RW, Elliott DE, Urban JF, Jr, Thompson RA, Weinstock JV.** 2005. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology **128**:825–832.
5. **Summers RW, Elliott DE, Urban JF, Jr, Thompson R, Weinstock JV.** 2005. *Trichuris suis* therapy in Crohn's disease. Gut **54**:87–90.
6. **Beer RJ.** 1976. The relationship between *Trichuris trichiura* (Linnaeus 1758) of man and *Trichuris suis* (Schrank 1788) of the pig. Res. Vet. Sci. **20**:47–54.
7. **Gaze S, McSorley HJ, Daveson J, Jones D, Bethony JM, Oliveira LM, Speare R, McCarthy JS, Engwerda CR, Croese J, Loukas A.** 2012. Characterising the mucosal and systemic immune responses to experimental human hookworm infection. PLoS Pathog. **8**:e1002520. doi:10.1371/journal.ppat.1002520.
8. **Daveson AJ, Jones DM, Gaze S, McSorley H, Clouston A, Pascoe A, Cooke S, Speare R, Macdonald GA, Anderson R, McCarthy JS, Loukas A, Croese J.** 2011. Effect of hookworm infection on wheat challenge in celiac disease: a randomised double-blinded placebo controlled trial. PLoS One **6**:e17366. doi:10.1371/journal.pone.0017366.
9. **Navarro S, Ferreira I, Loukas A.** 2013. The hookworm pharmacopoeia for inflammatory diseases. Int. J. Parasitol. **43**:225–231.
10. **Radford-Smith GL.** 2005. Will worms really cure Crohn's disease? Gut **54**:6–8.
11. **Khan WI, Blennerhasset PA, Varghese AK, Chowdhury SK, Omsted P, Deng Y, Collins SM.** 2002. Intestinal nematode infection ameliorates experimental colitis in mice. Infect. Immun. **70**:5931–5937.
12. **Elliott DE, Metwali A, Leung J, Setiawan T, Blum AM, Ince MN, Bazzone LE, Stadecker MJ, Urban JF, Jr, Weinstock JV.** 2008. Colonization with *Heligmosomoides polygyrus* suppresses mucosal IL-17 production. J. Immunol. **181**:2414–2419.
13. **Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF, Jr, Weinstock JV.** 2004. *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. Eur. J. Immunol. **34**:2690–2698.
14. **Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM.** 2005. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. J. Exp. Med. **202**:1199–1212.
15. **Hunter MM, Wang A, Parhar KS, Johnston MJ, Van Rooijen N, Beck PL, McKay DM.** 2010. In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. Gastroenterology **138**:1395–1405.
16. **Smith P, Mangan NE, Walsh CM, Fallon RE, McKenzie AN, van Rooijen N, Fallon PG.** 2007. Infection with a helminth parasite prevents experimental colitis via a macrophage-mediated mechanism. J. Immunol. **178**:4557–4566.
17. **Hewitson JP, Grainger JR, Maizels RM.** 2009. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. Mol. Biochem. Parasitol. **167**:1–11.
18. **Johnston MJ, MacDonald JA, McKay DM.** 2009. Parasitic helminths: a pharmacopeia of anti-inflammatory molecules. Parasitology **136**:125–147.
19. **Ruyssers NE, De Winter BY, De Man JG, Loukas A, Herman AG, Pelckmans PA, Moreels TG.** 2008. Worms and the treatment of inflammatory bowel disease: are molecules the answer? Clin. Dev. Immunol. **2008**:567314.
20. **Flynn RJ, Irwin JA, Olivier M, Sekiya M, Dalton JP, Mulcahy G.** 2007. Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immunol. Immunopathol. **120**:31–40.
21. **Wirtz S, Neufert C, Weigmann B, Neurath MF.** 2007. Chemically induced mouse models of intestinal inflammation. Nat. Protoc. **2**:541–546.
22. **Ruyssers NE, De Winter BY, De Man JG, Loukas A, Pearson MS, Weinstock JV, Van den Bossche RM, Martinet W, Pelckmans PA, Moreels TG.** 2009. Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice. Inflamm. Bowel Dis. **15**:491–500.
23. **Hong T, Yang Z, Lv CF, Zhang Y.** 2012. Suppressive effect of berberine on experimental dextran sulfate sodium-induced colitis. Immunopharmacol. Immunotoxicol. **34**:391–397.
24. **Donnelly S, O'Neill SM, Sekiya M, Mulcahy G, Dalton JP.** 2005. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect. Immun. **73**:166–173.
25. **Gordon S, Martinez FO.** 2010. Alternative activation of macrophages: mechanism and functions. Immunity **32**:593–604.
26. **McSorley HJ, Loukas A.** 2010. The immunology of human hookworm infections. Parasite Immunol. **32**:549–559.
27. **Geiger SM, Alexander ND, Fujiwara RT, Brooker S, Cundill B, Diemert DJ, Correa-Oliveira R, Bethony JM.** 2011. *Necator americanus* and helminth coinfections: further downmodulation of hookworm-specific type 1 immune responses. PLoS Negl. Trop. Dis. **5**:e1280. doi:10.1371/journal.pntd.0001280.
28. **Loukas A, Constant SL, Bethony JM.** 2005. Immunobiology of hookworm infection. FEMS Immunol. Med. Microbiol. **43**:115–124.
29. **Cuellar C, Wu W, Mendez S.** 2009. The hookworm tissue inhibitor of metalloproteases (Ac-TMP-1) modifies dendritic cell function and induces generation of CD4 and CD8 suppressor T cells. PLoS Negl. Trop. Dis. **3**:e439. doi:10.1371/journal.pntd.0000439.
30. **Moyle M, Foster DL, McGrath DE, Brown SM, Laroche Y, De Meutter J, Stanssens P, Bogowitz CA, Fried VA, Ely JA.** 1994. A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. J. Biol. Chem. **269**:10008–10015.
31. **Hsieh GC, Loukas A, Wahl AM, Bhatia M, Wang Y, Williamson AL, Kehn KW, Maruyama H, Hotez PJ, Leitenberg D, Bethony J, Constant SL.** 2004. A secreted protein from the human hookworm *Necator americanus* binds selectively to NK cells and induces IFN-gamma production. J. Immunol. **173**:2699–2704.
32. **Geiger SM, Fujiwara RT, Freitas PA, Massara CL, Dos Santos Carvalho O, Correa-Oliveira R, Bethony JM.** 2011. Excretory-secretory products from hookworm l(3) and adult worms suppress proinflammatory cytokines in infected individuals. J. Parasitol. Res. **2011**:512154.
33. **Elliott DE, Weinstock JV.** 2012. Helminth-host immunological interactions: prevention and control of immune-mediated diseases. Ann. N. Y. Acad. Sci. **1247**:83–96.
34. **Motomura Y, Wang H, Deng Y, El-Sharkawy RT, Verdu EF, Khan WI.** 2009. Helminth antigen-based strategy to ameliorate inflammation in an experimental model of colitis. Clin. Exp. Immunol. **155**:88–95.
35. **Johnston MJ, Wang A, Catarino ME, Ball L, Phan VC, MacDonald JA, McKay DM.** 2010. Extracts of the rat tapeworm, *Hymenolepis diminuta*, suppress macrophage activation in vitro and alleviate chemically induced colitis in mice. Infect. Immun. **78**:1364–1375.
36. **Cancado GG, Fiuza JA, de Paiva NC, Lemos LD, Ricci ND, Gazzinelli-Guimaraes PH, Martins VG, Bartholomeu DC, Negrao-Correa DA, Carneiro CM, Fujiwara RT.** 2011. Hookworm products ameliorate dextran sodium sulfate-induced colitis in BALB/c mice. Inflamm. Bowel Dis. **17**:2275–2286.
37. **McSorley HJ, Gaze S, Daveson J, Jones D, Anderson RP, Clouston A, Ruyssers NE, Speare R, McCarthy JS, Engwerda CR, Croese J, Loukas A.** 2011. Suppression of inflammatory immune responses in celiac disease by experimental hookworm infection. PLoS One **6**:e24092. doi:10.1371/journal.pone.0024092.
38. **Allen JE, Lawrence RA, Maizels RM.** 1996. APC from mice harbouring the filarial nematode, *Brugia malayi*, prevent cellular proliferation but not cytokine production. Int. Immunol. **8**:143–151.
39. **Menzies FM, Henriquez FL, Alexander J, Roberts CW.** 2010. Sequential expression of macrophage anti-microbial/inflammatory and wound healing markers following innate, alternative and classical activation. Clin. Exp. Immunol. **160**:369–379.
40. **Mulvenna J, Hamilton B, Nagaraj SH, Smyth D, Loukas A, Gorman JJ.** 2009. Proteomics analysis of the excretory/secretory component of the blood-feeding stage of the hookworm, *Ancylostoma caninum*. Mol. Cell. Proteomics **8**:109–121.
41. **Du L, Tang H, Ma Z, Xu J, Gao W, Chen J, Gan W, Zhang Z, Yu X, Zhou X, Hu X.** 2011. The protective effect of the recombinant 53-kDa protein of *Trichinella spiralis* on experimental colitis in mice. Dig. Dis. Sci. **56**:2810–2817.
42. **Schnoeller C, Rausch S, Pillai S, Avagyan A, Wittig BM, Loddenkemper C, Hamann A, Hamelmann E, Lucius R, Hartmann S.** 2008. A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. J. Immunol. **180**:4265–4272.
43. **Bhargava P, Li C, Stanya KJ, Jacobi D, Dai L, Liu S, Gangl MR, Harn DA, Lee CH.** 2012. Immunomodulatory glycan LNFPIII alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways. Nat. Med. **18**:1665–1672.